Subterranean Biology 44: 167–198 (2022)
doi: 10.3897/subtbiol.44.96545
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Free living ciliated protists from the chemoautotrophic cave ecosystem of Frasassi (Italy)

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Academic editor: R. Mayén-Estrada | Received 20 October 2022 | Accepted 17 December 2022 | Published 30 December 2022

https://zoobank.org/0F063098-7A0A-406C-8EA6-2F05A661532B

Citation: Bharti D, Kumar S, Buonanno F, Ortenzi C, Montanari A, Quintela-Alonso P, La Terza A (2022) Free living ciliated protists from the chemoautotrophic cave ecosystem of Frasassi (Italy). Subterranean Biology 44: 167–198. https://doi.org/10.3897/subtbiol.44.96545

Abstract

This study provides the first report on a community of free-living ciliated protists from the chemoautotrophic cave ecosystem of Frasassi, Italy. This subterranean groundwater ecosystem represents a hotspot of biodiversity that still needs to be fully explored with particular reference to microbial eukaryotes such as protist ciliates. A total of 33 taxa of ciliates were identified along with one species each of flagellate, heliozoans and naked amoebae, from four main sampling sites, namely, Grotta Solfurea (GSO), Lago Verde (LVE), Ramo Solfureo (RSO), and Pozzo dei Cristalli (PDC). The last consists of small microhabitats/ponds presenting different chemical–physical and biological parameters, such as sulfur and nutrient contents and the presence of bacterial biofilms. Furthermore, an analysis of the cryptic ciliate species biosphere as a 'seedbank' of diversity against cave ecosystem disturbance was also performed. This study also highlights some peculiar adaptations of cave-dwelling ciliates not described in their noncave-dwelling conspecifics, such as the extreme photosensitivity of *Urocentrum turbo*, the cannibalism of *Coleps hirtus*, the variable number of thorns in *Aspidisca* species as a defensive response to predation, and the frequent reorganization of ciliary structures in *Euplotes aediculatus*. The 18S rDNA sequences were generated for five species and were compared with those of the noncave-dwelling conspecifics. Finally, our results shed light on the still largely unknown ciliate diversity in the chemosynthesis-based sulfidic groundwater ecosystem of Frasassi.

Keywords
Adaptation, cannibalism, cryptic diversity, environmental stress, feeding behaviour, seed bank, sulphur

Introduction

Caves are unique subterranean habitats that have remained relatively stable, except for climatic fluctuations, for thousands of years. They are characterized by complete darkness, nearly constant air and water temperatures, high humidity near saturation, and poor supply of nutrients (Gale 1992). In this regard, cave organisms show a high capacity to thrive and adapt to these peculiar, as well as "extreme", environmental conditions (van Beynen and Townsend 2005; Galassi et al. 2017). Furthermore, these characteristics also suggest that cave organisms might be highly vulnerable to climatic changes and thus may become some of the rarest and most threatened species/biocenoses on Earth (van Beynen and Townsend 2005; Galassi et al. 2017). Survival in these habitats requires specific adaptations for tolerating the stress of living in darkness and other extreme environmental conditions, such as nutrient and energy limitations; relatively low but stable temperatures (within a range from 13.0 to 13.5 °C); toxic levels of gases (e.g., H_2S, CO_2, CH_4); low oxygen concentrations; and variable hydrogen ion concentrations. In this context, the chemoautotrophic cave ecosystem of Frasassi located in the Marche region of central Italy, on the eastern side of the Apennine Mountains, represents one of the most investigated sulfidic cave ecosystems around the world (Galdenzi and Jones 2017). In this hypogenic cave system, Galdenzi et al. (1997) reported the presence of biofilms, signifying that bacterial communities play an important role in hydrogen sulfide oxidation. Moreover, Galdenzi et al. (1999) observed the growth of microbial biofilms, which secrete sulfuric acid (H_2SO_4) with a pH < 1, known as mucolites. Later, Vlasceanu et al. (2000) found that sulfur-oxidizing bacteria on biofilms play an important role in limestone corrosion and are at the base of the cave trophic web.

In addition to bacteria and archaea, several other cave-dwelling organisms, mainly represented by invertebrates, have been identified and described from the Frasassi cave. In the past two decades, independent faunal surveys in the Frasassi cave complex have led to the discovery of several invertebrate species, including some new to science. The list includes mainly Crustacea (Ostracoda, Copepoda, and Amphipoda; Bertolani et al. 1994; Flot et al. 2010; Karaman et al. 2010; Peterson et al. 2013), Mollusca (Gastropoda; Bodon and Cianfanelli 2012), and Platyhelminthes (Stocchino et al. 2017). However, apart from invertebrates, among the different organism types inhabiting cave systems across the world, it is worth mentioning unicellular, eukaryotic ciliated protists. Since the 19th century, cave-dwelling protists have been an interesting, although challenging, topic for researchers. To date, due to the difficulties in sampling in such harsh environments, very few studies have been performed to describe ciliate communities in caves (Gittleson and Hoover 1969, 1970; Sudzuki and Hosoyama 1991; Landolt et al. 1992; Golemansky and Bonnet 1994; Copellotti and Guidolin 1999,

2003; De Luca et al. 2005). Therefore, ciliates have remained largely unexplored, especially those dwelling in karst environments.

Apart from diversity, some morphological, behavioural and physiological adaptations to life in cave systems have also been reported. In particular, Copellotti and Guidolin (1999) found that ciliate species recorded from karst caves in northeastern Italy show unique morphological characteristics (e.g., increase in the number of contractile vacuoles, or body size ratio reduced to almost 30%), and their communities are expected to be more diverse (at least from a morphological point of view) than those of their noncave-dwelling conspecifics as a result of the long-term adaptation to these highly stressful ecosystems. Intraspecific aggressiveness, as an adaptation to maintain populations in resource-depleted environments such as caves or ephemeral habitats, has been reported in several groups, such as arachnids (spiders), crustaceans (hermit crabs), amphibians (toads and salamanders) and reptiles (lizards) (Arnott and Elwood 2008; Cooper et al. 2015; Melotto et al. 2019). However, these morphological and behaviour traits have been scantly studied in the realm of microorganisms such as protists.

The present investigation aims to characterize for the first time the diversity of cave-dwelling ciliated protists from the Frasassi cave ecosystem in the Marche region (Italy; Fig. 1), including the gene sequence (18S rRNA gene) of six species, and to describe some of the morphological and behavioural differences with their noncave-dwelling counterpart ciliate species.

Materials and methods

Sampling locations

Four main sampling locations were selected in the Frasassi cave system (coordinates WGS84-G: 43.402°N, 12.962°E), which are Grotta Solfurea (GSO), Lago Verde (LVE), Ramo Solfureo (RSO) and Pozzo dei Cristalli (PDC) (in Grotta del Fiume Cave) (Fig. 1A, B). The sampling sites were selected on the basis of the presence of sulfidic water pools or springs and were accessed via technical spelunking routes.

The ciliate diversity from site PDC was studied in detail since it is highly diversified and includes seven distinct microhabitats [i.e. Strettoia del Tarlo (SDT), Lago della Scala (LDS), Pozzo dei Cristalli Stream (PCS), Pozzo dei Cristalli Pond (PCP), Hydrogen Sulfide Spring (HSS), Lago Galdenzi (LGA), and Lago della Bottiglia (LDB)], represented by small sulfidic (H_2S-rich) ponds, streams, and springs as well as deep and shallow muddy, stagnant lakes (Fig. 1B). However, the topological configuration of these sites/microhabitats, and in particular of the highly diversified PDC site, which is located in the close proximity of the Sentino River (which was also sampled with recording of *Urocentrum turbo* noncave-dwelling population), can be dramatically altered following heavy rainfall events that may provoke an increase in water levels inside the cave, resulting in merging all the PDC microhabitats (Fig. 1). For this reason, sites

were accessed exclusively during "dry periods" (i.e., far from heavy rainfall events) to collect water/sediment samples in similar ecological conditions across different sampling dates and to make easier access to the different sites. Since the main objective of this study was to perform a study on cave-dwelling protist communities from the Frasassi cave complex, no chemical–physical parameters were measured. Table 1 lists some of the main geochemical parameters (H_2S and O_2 concentrations) recorded by other researchers during the most accessible dry season (Dattagupta et al. 2009; Flot et al. 2010; Bauermeister et al. 2012).

The Italian noncave-dwelling population of *Urocentrum turbo* was isolated in September 2011 from small ponds near the bank of Sentino River, which flows in the deep Frasassi Gorge (43°24'03"N, 12°57'55"E) (Fig. 1A). The Indian noncave-dwelling populations of *Urocentrum turbo* were isolated from water samples collected in July 2021 from a local pond in Khadabandha (21°57'08"N, 85°23'35"E), Odisha, India, and in October 2021 from Khajjiar Lake (32°32'48"N, 76°03'26"E; Ramsar site), Himachal Pradesh, India. The Indian noncave-dwelling population of *Coleps hirtus* was isolated in December 2018 from a natural small pond near the bank of the Ganges River (Hooghly), Kolkata (22°29'26"N, 88°12'28"E). The water samples were transferred to the laboratory at the Zoological Survey of India, Kolkata, for further processing according to Bharti et al. (2019).

Table 1. Main Geochemical parameters of PDC, GSO, LVE, and RSO (Dattagupta et al. 2009; Flot et al. 2010; Bauermeister et al. 2012).

Site description	Collection date	[O2] (μM)	[H2S] (μM)
PDC	August 2006	0.2	322
	May 2007	2.5	542
	May-June 2009	12	415
GSO	May 2007	1.2	201
	May-June 2009	51	118
LVE	May 2007	3.6	301
	May-June 2009	2	415
RSO	August 2006	1.0	195
	May 2007	1.6	240
	May-June 2009	10	109

Ciliate sampling and processing

Periodic sampling was carried out from 2009 to 2011 (Table 2). However, and mainly due to accessibility constraints, it was not always possible to sample all sites during each sampling date since most of these sites were accessible exclusively during the dry season. Water and sediment were collected in half-filled 250 ml sterile plastic bottles and maintained at a temperature close to that of the sampling site by using a 12 V portable refrigerator (GEOSALD SRL, Milan, Italy) until they reached the laboratory at the University of Camerino, Italy (approximately 45 min drive from Frasassi).

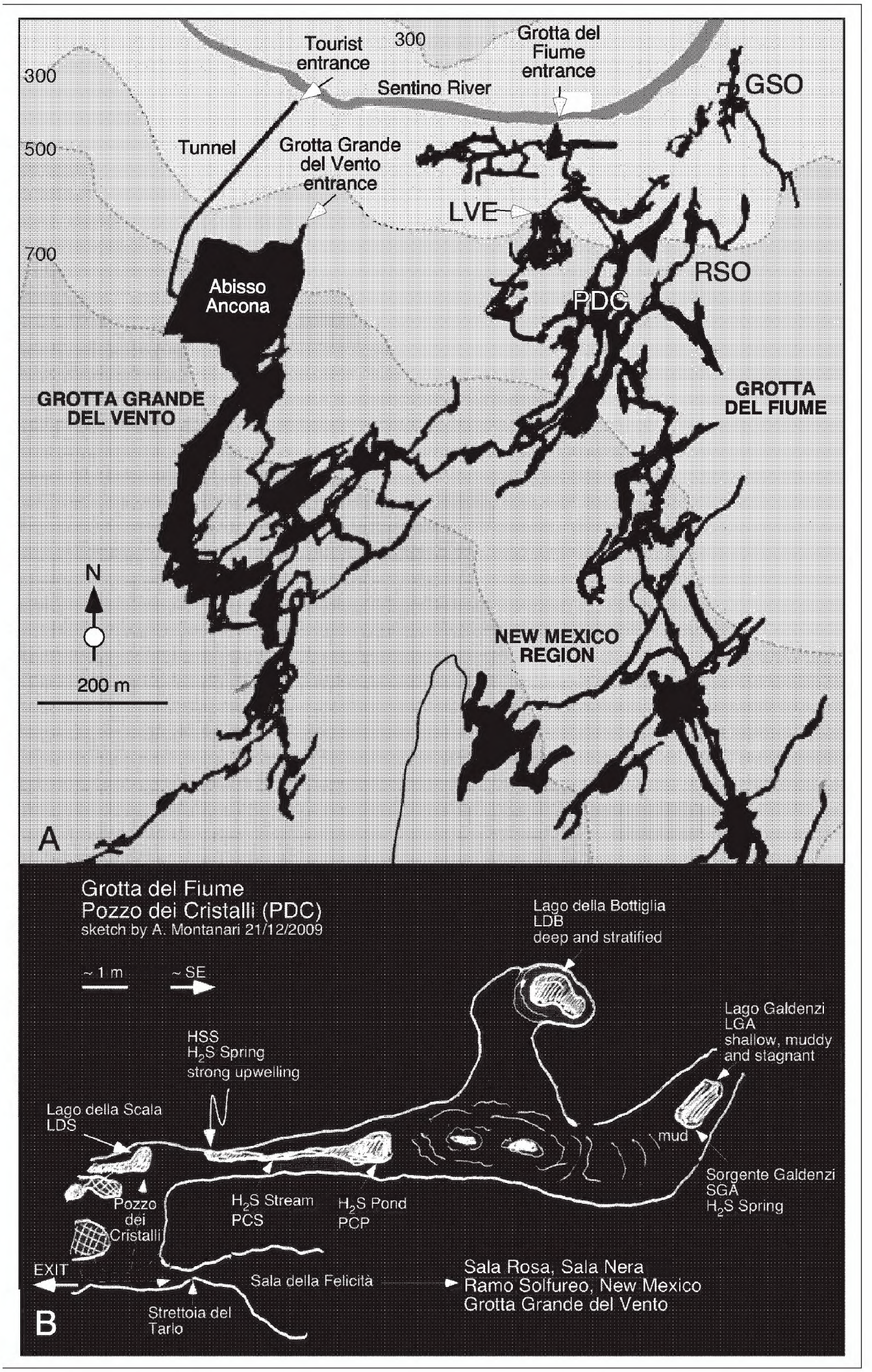

Figure 1. A map of the Frasassi cave system showing the location of the main four sampling sites (Map modified after Mariani et al. (2007) **B** simplified map of the PDC showing the seven microhabitats (Drawn by Dr. Alessandro Montanari).

Nytex nets of decreasing mesh size (500 to 150 μm) were used in succession to filter out crustaceans, sand grains, and other unwanted materials. In the laboratory, all ciliate cell cultures were maintained in a dark chamber at 13 °C. To investigate the possible presence of cryptic ciliate species, each sample was split into two Petri dishes, one containing autoclaved rice/wheat grain to support bacterial growth and the other with the green alga *Chlorogonium elongatum.* These samples were periodically checked for a period of 20 days for the presence of ciliates. Live observations were made using a microscope with bright-field illumination at a magnification of 100–1000×. An Optika microscope camera was employed for photomicrography and video captures. Protargol staining (Kamra and Sapra 1990) and silver nitrate staining (Corliss 1953) were employed to reveal the infraciliature and cortical structures. Classification is according to Lynn (2008).

Table 2. Sampling sites and calendar for collection of water and sediment samples from 2009 to 2011. + indicates sampling performed.

Sampling sites/dates	PDC							LVE	RSO	GSO
	SDT	PCP	PCS	HSS	LDS	LGA	LDB			
Oct 2009		+	+	+	+	+	+			
Dec 2009					+					
Feb 2010	+									
March 2010	+	+	+	+				+		
April 2010	+									
Sept 2011		+	+	+	+	+	+	+		
Oct 2011									+	+
Total	3	3	3	3	3	2	2	2	1	1

DNA extraction, PCR amplification, cloning and sequencing

DNA extraction was performed according to Thomas et al. (2005). Five to six cells were collected with the help of glass micropipettes and washed three times with autoclaved distilled water. For DNA extraction, 50 μl of 5% (w/v) Chelex 100 with 2 μl of Proteinase K (20 mg/ml) solution was added to the sample and then incubated at 37 °C for 30 minutes, followed by incubation at 98 °C for 5 minutes. The reaction mixture was cooled immediately on ice and centrifuged in a microfuge tube for 2–3 seconds at a speed of 16,000 rpm. Without picking the Chelex100 beads, 2 μl of the supernatant was carefully drawn from the top and stored at 4 °C until PCR amplification. Extracted DNA (2 μl) was dispensed into a PCR tube containing 5 μl of distilled water; amplifications by PCR were carried out in a total volume of 50 μl containing 10× PCR buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 mM of each oligonucleotide primer, and 5 U of Taq DNA polymerase (USB). PCR amplification was performed with the universal eukaryotic primers (Medlin et al. 1988) Euk A, 5'-AACCTGGTTGATCCTGCCAGT-3' (forward primer), and Euk B, 5'-TGATCCTTCTGCAGGTTCACCTAC- 3' (reverse primer). Additionally, the nested primer

pairs *Euplotes* 18S (FW 5'-TAG AGG GAC TTT GTG TGC AAC C-3') and *Euplotes* 18S (RV 5'-ATC TCC CTG AAA CAC ACG TTG G-3') were used in combination with universal primers for partial 18S rDNA amplification. The PCR program included initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 1 minute, 55 °C for 45 seconds, and 72 °C for 80 seconds with a final extension step at 72 °C for 10 minutes. After confirmation of appropriate size by agarose gel electrophoresis, PCR products were purified using the Nucleospin gel extraction kit (Qiagen, Milan, Italy) and inserted into a PGEM-T easy vector system (Promega, Milan, Italy). Plasmids showing the right size inserts (i.e., in a range of 1600–1800 bp) were purified and sequenced at either BMR genomics (Italy) or STAR Seq (Germany).

Results

Ciliate community composition and species richness

In the present study, 36 protist taxa from four different sampling sites within the Frasassi cave system, consisting of 33 ciliate species, one flagellate species, one naked amoeba species and one heliozoan species, were recorded. Flagellates, belonging to the genus *Peranema*, were present in the LDS, HSS and PCP sampling sites, while amoebas were present in the LGA sample, and heliozoans were present in the SDT sampling site.

The 33 identified species of ciliates belong to 8 classes, 15 orders and 25 families (Table 3, Figs 2–6). Two classes contributed more than half of the total species recorded, i.e., class Spirotrichea (11 species belonging to 3 orders and 6 families), with a dominance of 0.35, and class Oligohymenophorea (6 species belonging to 3 orders and 5 families), with a dominance of 0.19 (Tables 3, 4, Figs 2–5). The highest diversity of ciliates was found in PDC (32 species and 24 families), followed by GSO (11 species belonging to 10 orders and 11 families), LVE (5 species belonging to 5 orders and 5 families) and RSO (2 species belonging to 2 orders and 2 families). Peniculida and Sporadotrichida were the only orders common to the four sampling locations, while Colpodida, Haptorida, Heterotrichida, Hymenostomatida and Urostylida were only present in PDC, and Armophorida was only recorded in GSO. Oxytrichidae was the only family present in the four locations (Tables 3, 4).

Ciliate species richness in PDC, considering the complete sampling campaign from 2009–2011 (Table 2), consisted of 32 species, with the maximum number of species recorded at sites PCP (17 species) and SDT (16 species) (Table 3). Site PCP had species that were present in the other sites of PDC; however, some of the species were exclusively reported from site SDT, i.e., *Climacostomum virens*, *Spirostomum ambiguum*, *Tachysoma pellionellum*, *Urostyla* sp. and *Vorticella picta*. The LDS site had the third highest species richness value (13 species), followed by LGA and PCS (11 and 10 species, respectively). The species *Paracolpoda steinii* was exclusively found in LGA, and *Anteholosticha monilata* was found only in LDS. The lowest species richness values were recorded for sites HSS and LDB, with 8 and 6 species, respectively (Table 3).

Table 3. Ciliated Protozoa from the sampling sites of Frasassi caves. Species in bold are the first report for the Italian caves. + indicates presence of the species.

S. No.	Species	Class	Order	Family	PDC							LVE	RSO	GSO
					SDT	LDS	HSS	PCS	PCP	LGA	LDB			
1	*Anteholosticha monilata* (Kahl, 1932) Berger, 2003	Spirotrichea	Urostylida	Holostichidae		+								
2	***Anteholosticha sigmoidea*** (Foissner, 1982) Berger, 2003	Spirotrichea	Urostylida	Holostichidae	+				+					
3	*Aspidisca turrita* (Ehrenberg, 1831) Claparède & Lachmann, 1858	Spirotrichea	Euplotida	Aspidiscidae		+		+	+	+				+
4	*Brachonella* sp.	Armophorea	Metopida	Metopidae		+			+					+
5	***Caenomorpha* sp.**	Armophorea	Armophorida	Caenomorphidae										+
6	*Chilodonella uncinata* (Ehrenberg, 1838) Strand, 1928	Phyllopharyngea	Chlamydodontida	Chilodonellidae	+			+	+					+
7	***Climacostomum virens*** (Ehrenberg, 1838) Stein, 1859	Heterotrichea	Heterotrichida	Climacostomidae	+									
8	*Coleps hirtus* (Müller, 1786) Nitzsch, 1827	Prostomatea	Prorodontida	Colepidae				+	+	+	+	+		+
9	*Colpoda inflata* (Stokes, 1884) Kahl, 1931	Colpodea	Colpodida	Colpodidae						+	+			
10	***Cyrtolophosis mucicola*** Stokes, 1885	Colpodea	Cyrtolophosidida	Cyrtolophosididae	+	+	+	+	+	+	+	+		+
11	*Dileptus* sp.	Litostomatea	Haptorida	Dileptidae				+	+	+				
12	***Euplotes aediculatus*** Pierson, 1943	Spirotrichea	Euplotida	Euplotidae		+								+
13	*Euplotes* sp.	Spirotrichea	Euplotida	Euplotidae	+	+	+	+	+	+				
14	*Frontonia leucas* (Ehrenberg, 1833) Ehrenberg, 1838	Oligohymenophorea	Peniculida	Frontoniidae	+		+							
15	*Gonostomum affine* (Stein, 1859) Sterki, 1878	Spirotrichea	Sporadotrichida	Gonostomatidae	+	+								
16	***Lacrymaria* sp.**	Litostomatea	Haptorida	Lacrymariidae		+	+		+					
17	*Litonotus lamella* (Müller, 1773) Foissner et al. 1995	Litostomatea	Pleurostomatida	Litonotidae		+	+		+	+				+
18	***Oxytricha setigera*** Stokes, 1891	Spirotrichea	Sporadotrichida	Oxytrichidae			+		+					
19	*Oxytricha* sp.	Spirotrichea	Sporadotrichida	Oxytrichidae		+	+		+		+	+	+	+

S. No.	Species	Class	Order	Family	PDC							LVE	RSO	GSO
					SDT	LDS	HSS	PCS	PCP	LGA	LDB			
20	*Paracolpoda steinii* (Maupas, 1883) Lynn, 1978	Colpodea	Colpodida	Colpodidae						+				
21	*Paramecium caudatum* Ehrenberg, 1833	Oligohymenophorea	Peniculida	Parameciidae	+		+						+	
22	***Paruroleptus* sp.**	Spirotrichea	Urostylida	Urostylidae	+				+					
23	***Pelagothrix* sp.**	Prostomatea	Prorodontida	Holophryidae				+		+				
24	*Spathidium* sp.	Litostomatea	Haptorida	Spathidiidae					+	+				
25	***Spirostomum ambiguum*** (Müller, 1786) Ehrenberg, 1835	Heterotrichea	Heterotrichida	Spirostomidae	+									
26	***Stentor polymorphus*** (Müller, 1773) Ehrenberg, 1830	Heterotrichea	Heterotrichida	Stentoridae	+	+								
27	***Tachysoma pellionellum*** (Müller, 1773) Borror, 1972	Spirotrichea	Sporadotrichida	Oxytrichidae	+						+			
28	*Tetrahymena pyriformis* (Ehrenberg, 1830) Lwoff, 1947	Oligohymenophorea	Hymenostomatida	Tetrahymenidae				+	+		+			
29	***Trithigmostoma* sp.**	Phyllopharyngea	Chlamydodontida	Chilodonellidae	+	+								
30	***Urocentrum turbo*** (Müller, 1786) Nitzsch, 1827	Oligohymenophorea	Peniculida	Urocentridae	+			+	+			+		+
31	***Urostyla* sp.**	Spirotrichea	Urostylida	Urostylidae	+									
32	*Vorticella picta* (Ehrenberg, 1831) Ehrenberg, 1838	Oligohymenophorea	Sessilida	Vorticellidae	+									
33	*Vorticellides aquadulcis* (Stokes, 1887) Foissner et al. 2010	Oligohymenophorea	Sessilida	Vorticellidae		+		+	+	+		+		+
	Total numbers	**8**	**15**	**25**	**16**	**13**	**8**	**10**	**17**	**11**	**6**	**5**	**2**	**11**

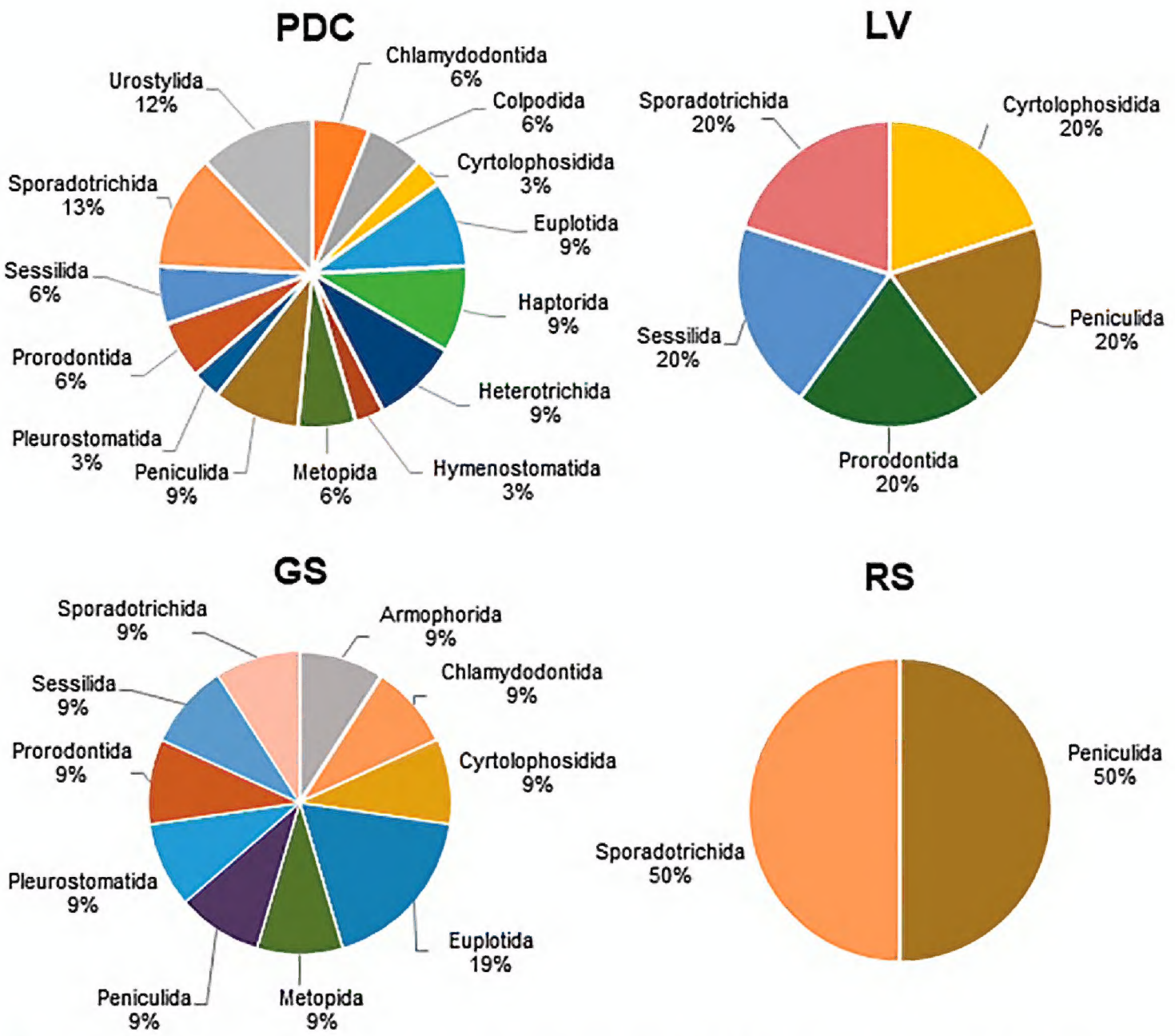

Figure 2. Percentage of each ciliate order in four locations of the Frasassi cave system to show communities assemblages from 2009 to 2011.

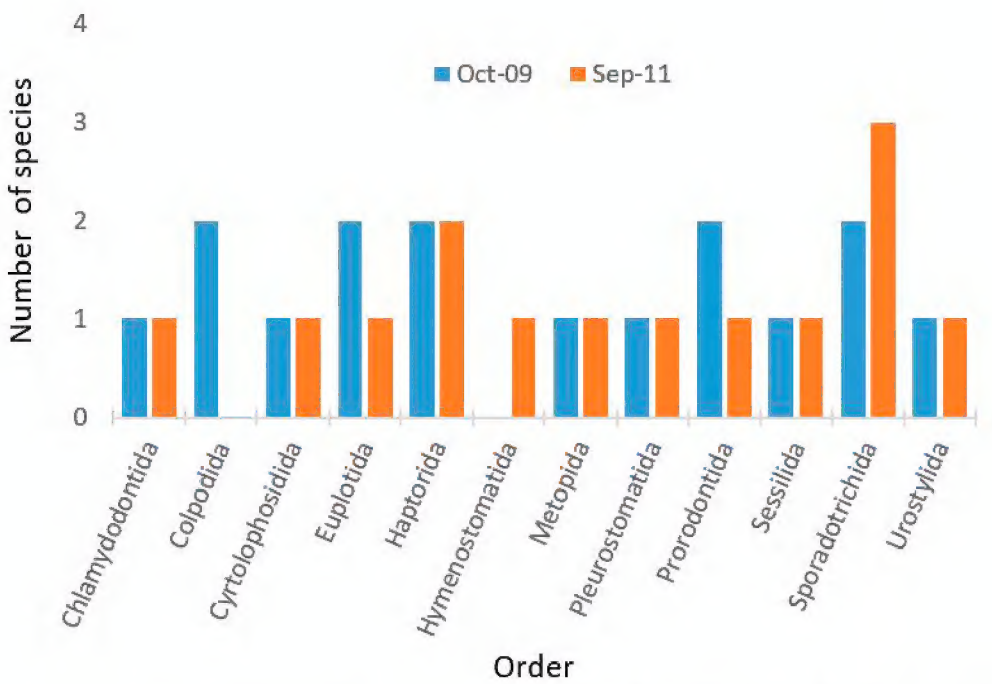

Figure 3. The comparative number of species of each ciliate order in PDC in October 2009 and September 2011.

The distribution of ciliates in PDC was investigated in greater detail for the samples collected in October 2009 and September 2011. In fact, and exclusively on these two sampling dates, it was possible to collect samples from all the investigated microhabitats to eventually observe differences in the community structure (Table 5, Fig. 3). A total of 18 species belonging to 12 orders and 15 families were recorded in PDC in these months. The highest species richness within PDC was found at sites LGA in October 2009 and PCP in September 2011, both with 11 species, although the ciliate communities showed a different species composition on both sampling occasions (Table 5, Figs 3–7). Thus, only three species were present in PCP during both samplings, i.e., *Brachonella* sp., *Oxytricha setigera*, and *Paruroleptus* sp., while no species were detected in the samples taken in September 2011 in LGA. A similar situation occurred in the other sampling sites of PDC, where only a few species from each site were present in both samplings, i.e., *Brachonella* sp. and *Oxytricha* sp. in LDS; *Chilodonella uncinata*, *Cyrtolophosis mucicola* and *Vorticellides aquadulcis* in PCS; and *Coleps hirtus* and *Cyrtolophosis mucicola* in LDB. The HSS, LDS, PCS, and LDB sites presented 4, 5, 6 and 6 ciliate species, respectively, with only one species, namely, *Cyrtolophosis mucicola*, common to the four sites (Table 5, Figs 4–7). Despite the differences in the ciliate species composition recorded in October 2009 and September 2011, the ciliate community structure in PDC at the order level remained mostly stable, i.e., from the 12 orders present in PDC on these dates, only two orders were present on one sampling occasion: Colpodida in October 2009 and Hymenostomatida in September 2011 (Fig. 3).

Most of the ciliate species identified in this study can use bacteria as a food resource, which explains why bacterivores, phytobacterivores and nonselective omnivores were the main trophic groups in the Frasassi cave system (Table 4). The presence of other trophic groups was restricted to PDC, and in particular to the sites PCP and LGA. This is likely associated with a greater offer of food sources within these microhabitats, which can host a higher diversity of ciliates than the other sites within PDC, mostly colonized by bacterial feeders. Thus, in PCP and LGA, specialized feeders such as the predator *Dileptus* sp. and *Spathidium* sp. were both found in PCP in October 2009 and in LGA in September 2011. Histophages such as *Pelagothrix* sp. in LGA were found in September 2011. A special mention should be made of the presence of the nonselective heterotrophic omnivore *Coleps hirtus*, identified in PCP in October 2009 and in LGA in September 2011. This species is a voracious feeder with a broad food spectrum that in this study in the Frasassi caves also exhibited a cannibalistic behaviour on other conspecific living individuals; never observed, to the best of our knowledge, before in noncave-dwelling counterpart congeners (Tables 4, 5).

Considering PDC as a single sampling site, its ciliate community was composed of 16 species in October 2009 and 14 species in September 2011, with a low species similarity between both samplings, since only a few species were recorded on both occasions, i.e., *Brachonella* sp., *Chilodonella uncinata*, *Coleps hirtus*, *Cyrtolophosis mucicola*, *Oxytricha setigera*, *Oxytricha* sp., *Paruroleptus* sp., and *Vorticellides aquadulcis* (Tables 2–5).

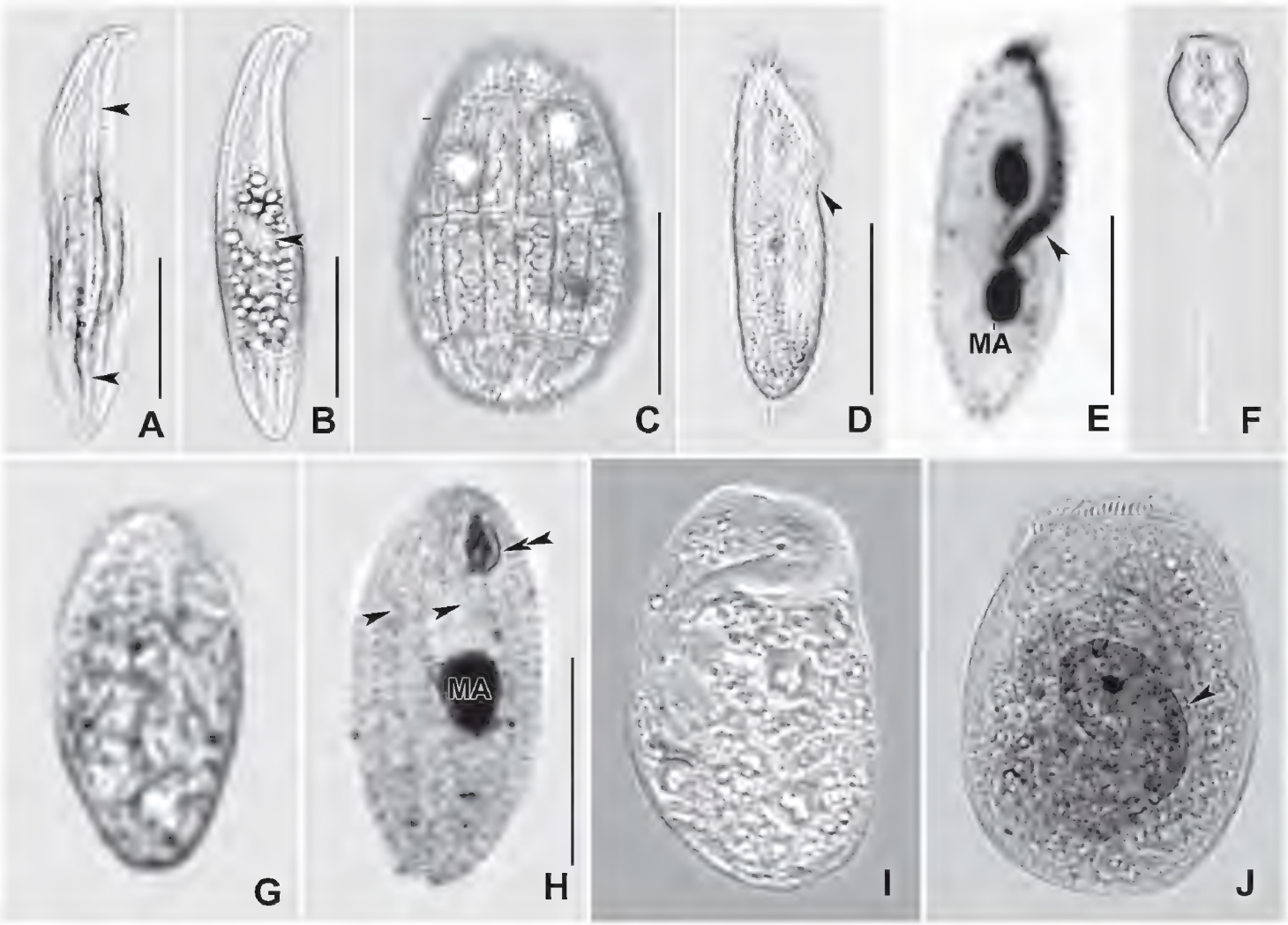

Figure 4. A, B Two specimens of *Litonotus lamella*, arrowheads mark the firm pellicle lines in **A** and the macronucleus in **B**. **C** *Coleps hirtus* **D, E** *Gonostomum affine* cells, arrowheads show the curved adoral zone of membranelles typical of the genus **F** *Vorticella picta* **G, H** live and protargol stained cells of *Tetrahymena pyriformis*, showing the oral apparatus with three adoral membranelles and paroral membrane (double arrowhead) and two of the ciliary rows (arrowheads) **I, J** representative specimens of *Climacostomum virens*, arrowhead in **J** marks the stained macronucleus. **MA** = macronuclear nodule. Scale bars: 25μm.

Cryptic biodiversity

The formation of dormant forms [e.g., resting cysts followed by reversible cytodifferentiation (cryptobiosis)] is a key adaptive strategy frequently adopted by ciliates to persist in harsh ecosystems, such as that represented by the chemoautotrophic cave ecosystem of Frasassi. Cryptic ciliate species biodiversity was also recovered by enriching the freshly collected water samples with autoclaved wheat or rice grains to stimulate bacterial growth and to produce a source of food for the "awakening" of the ciliate cyst dormant stages. Accordingly, while the number of ciliate species was low on freshly collected and observed samples (Table 6), the species richness gradually increased after the enrichment of the cultures. Observation of enriched samples on Days 3, 5 and 7 showed the excystment of dormant forms, thereby increasing the species richness. Cryptic species often recovered on successive days were *Chilodonella uncinata*, *Dileptus* sp., *Litonotus lamella*, *Oxytricha setigera*, *Oxytricha* sp., *Paruroleptus* sp., *Tachysoma pellionellum*, *Tetrahymena pyriformis* and *Vorticellides aquadulcis*.

Table 4. Taxonomic and trophic groups in the Frasassi caves from 2009 to 2011.

S. No.	Species	Trophic group	**Food preferences** (Foissner 1997; Foissner et al. 1999)
1	*Anteholosticha monilata*	Phytobacterivore	B, A, D, C
2	***Anteholosticha sigmoidea***	Bacterivore	B, Fungal spores
3	*Aspidisca turrita*	Bacterivore	B
4	*Brachonella* sp.	Bacterivore	B
5	***Caenomorpha* sp.**	Bacterivore	B, Sb
6	*Chilodonella uncinata*	Phytobacterivore	B, A, D
7	***Climacostomum virens***	Non-selective omnivore	B, A, D, Fl, Ta, C, R
8	*Coleps hirtus*	Non-selective omnivore	B, A, Fl
9	*Colpoda inflata*	Bacterivore	B, Fl
10	***Cyrtolophosis mucicola***	Bacterivore	B
11	*Dileptus* sp.	Predator	P, M
12	***Euplotes aediculatus***	Non-selective omnivore	O
13	*Euplotes* sp.	Non-selective omnivore	B, A, D, Fl
14	*Frontonia leucas*	Non-selective omnivore	O
15	*Gonostomum affine*	Non-selective omnivore	B, Fl, Dt
16	***Lacrymaria* sp.**	Predator	P, M
17	*Litonotus lamella*	Non-selective omnivore	Fl, C
18	***Oxytricha setigera***	Bacterivore	B, Fl
19	*Oxytricha* sp.	Bacterivore	B, Fl
20	*Paracolpoda steinii*	Bacterivore	B
21	*Paramecium caudatum*	Phytobacterivore	B, A
22	***Paruroleptus* sp.**	Non-selective omnivore	O
23	***Pelagothrix* sp.**	Histophage	H,
24	*Spathidium* sp.	Predator	P
25	***Spirostomum ambiguum***	Phytobacterivore	B, Sb, A, D
26	***Stentor polymorphus***	Non-selective omnivore	Fl, C
27	***Tachysoma pellionellum***	Strict algivore	B, Cy, A, D
28	*Tetrahymena pyriformis*	Bacterivore	B
29	***Trithigmostoma* sp.**	Strict algivore	A, D
30	***Urocentrum turbo***	Phytobacterivore	B, D
31	***Urostyla* sp.**	Phytobacterivore	B, D
32	*Vorticellides aquadulcis*	Bacterivore	B
33	*Vorticella picta*	Bacterivore	B

A = algae; **Am** = amoeba; **B** = bacteria; **C** = ciliates; **Cy** = cyanobacteria; **D** = diatoms; **Dt** = detritus; **Fl** = flagellates; **H** = histophage; **M** = metazoan; **O** = omnivore; **P** = protists; **R** = rotifers; **Sb** = sulphur bacteria; **Ta** = testate amoeba.

Adaptations of some ciliate species in cave environments

Some of the species isolated from the Frasassi caves exhibit some peculiar adaptations that clearly distinguish them from their conspecific, noncave-dwelling, populations. In addition, some species could not be cultured in the laboratory due to their particular and/or demanding culture conditions, e.g., a population of *Urocentrum turbo* isolated from the PDC (microhabitats: SDT, PCS and PCP), LVE and GSO sampling sites was found to be so extremely sensitive to light that even when observed in very low light conditions, the shape of the cells began to distort and eventually burst (Fig. 7B). Conversely, three populations of noncave-dwelling *Urocentrum turbo* isolated from the Sentino River,

Genga, Italy; a high altitude wetland in Himachal Pradesh, India; and a local pond in Odisha, India did not show any sensitivity to light exposure (Fig. 7). These cave- and noncave-dwelling populations also differ with respect to the presence or absence of peculiar extrusive organelles generally known as extrusomes with defensive/offensive functions (e.g., trichocysts, toxicysts), which are absent in the Sentino River population but present in both the Indian and cave populations. Moreover, no differences were found at the level of 18S rRNA gene sequences between the cave-dwelling *U. turbo* populations and their noncave-dwelling populations available in the NCBI database (Table 7).

The peculiar adaptations to the harsh cave ecosystem were observed in the ciliates *Aspidisca turrita*, *Euplotes aediculatus*, and *Coleps hirtus.* It has been reported that some morphological traits (e.g., furrows, spurs, dorsal ribs, thorns, etc.) may vary in number or size under different growth conditions (Copellotti and Guidolin 1999, 2003). This is the case for *Aspidisca turrita*, which exhibited a variable number of dorsal spines, ranging from one to two or three spines, and *Euplotes aediculatus*, which showed reorganization of the oral apparatus, ventral and dorsal ciliature. In contrast to the "regular" division where two sets of ciliature are formed, the reorganizer shows the formation of a single set of organelles. Our observations show that nearly 20 out of 100 specimens analysed were reorganizers (Fig. 8).

Faced with the impossibility of making resting cysts to survive adverse conditions (starvation, drying, etc.), some species have developed other adaptations, both morphological and behavioural, to survive in harsh environments. An example of these adaptations was observed in the worldwide distributed freshwater ciliate *Coleps hirtus*, a prostomatid ciliate with protective, calcified armour, as well as the ability to inject cytotoxic substances able to immobilize potential prey or predators *via* extrusomes, which hinders the survival of other competitors in the environment (Buonanno et al. 2014). While *Coleps hirtus* is generally known as a histophagous and scavenger feeder, the populations of this species found in the Frasassi caves also exhibited a cannibalistic behaviour on other conspecific living individuals never observed before in noncave-dwelling counterpart congeners (Fig. 9 and Suppl. materials 1–4).

SSU rRNA Gene Sequences and characteristics

Through this study, the small subunit rRNA gene sequences of five newly sequenced cave-dwelling ciliate isolates were obtained and blasted in the NCBI database, and the maximum similarity percent with noncave-dwelling species was determined, reported in Table 7, together with the assigned GenBank accession numbers. In particular, the 18S rRNA gene sequence of the cave-dwelling *Vorticella picta* showed the highest identity (98%) with *V. fusca* (DQ190468), whereas that of *Euplotes* sp. had the highest identity (99%) with that of *E. elegans* (DQ309868). Furthermore, the 18S rRNA sequence of the ciliate species identified as *Coleps hirtus hirtus* in a previous study (Buonanno et al. 2014) had 98% similarity with both sequences of *C. viridis* (MT253681) and *C. hirtus* (MT253687). The 18S rRNA gene length and GC content of the six species were as follows: *Coleps hirtus* (1775 bp in length and CG content of 45.1%), *Climacostomum virens* (1711 bp, 47.6%), *Euplotes aediculatus* (1874 bp, 44.3%), *Euplotes* sp. (1600 bp, 42.6%), *Vorticella picta* (1712 bp, 42.2%), and *Urocentrum turbo* (1660 bp, 42.8%).

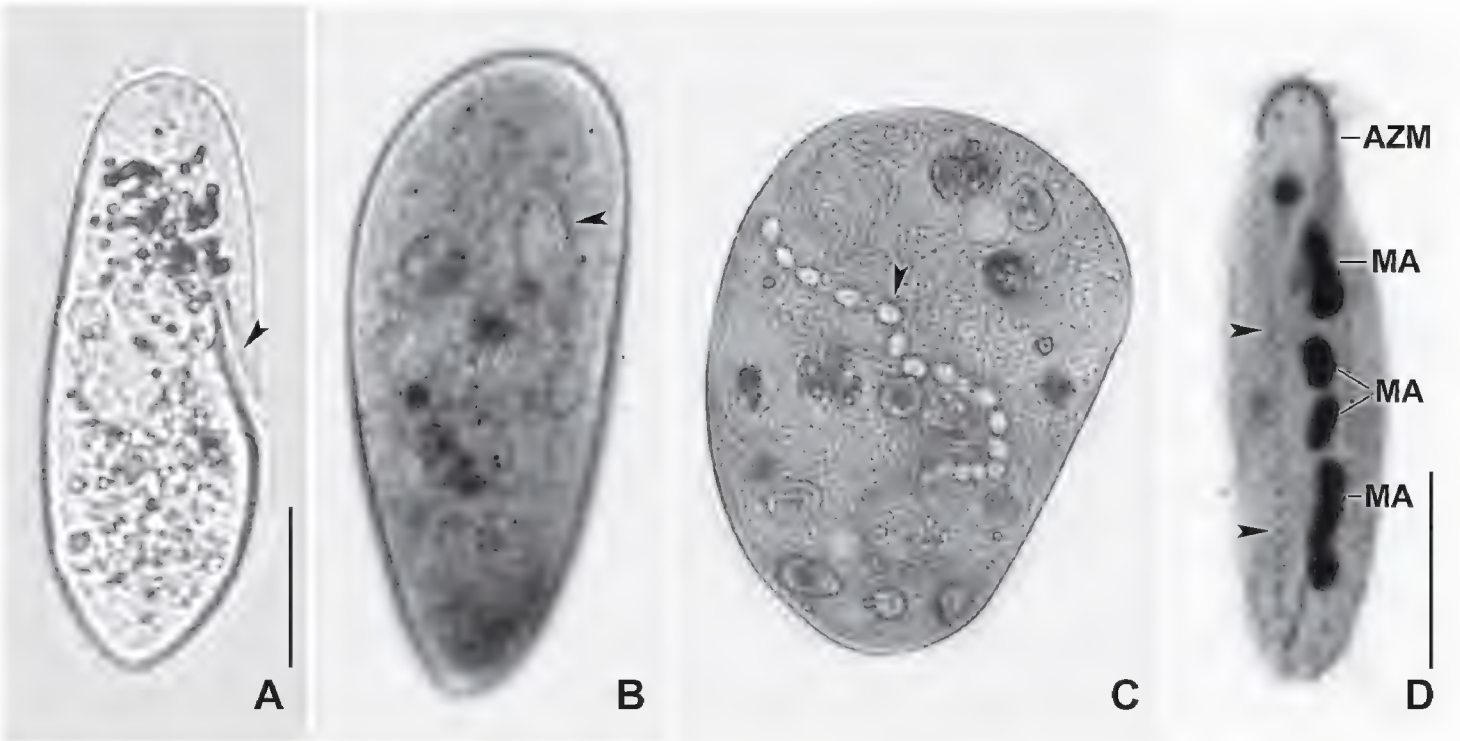

Figure 5. Photomicrographs of live **A–C** silver impregnated cells **D** from RSO **A** and PDC **B–D** *Paramecium caudatum* **A** and *Frontonia leucas* **B** arrowheads show the cytostomes **C** contracted specimen of *Stentor polymorphus*, arrowhead marks the moniliform macronucleus **D** protargol impregnated specimen of *Anteholosticha monilata* showing the zig-zag cirral row (arrowheads) of fronto-ventral cirri typical of the urostyloid group. **AZM** = adoral zone of membranelles; **MA** = macronuclear nodule. Scale bars: 40μm.

Discussion

Ciliate community structure: species richness, taxonomic and trophic groups

To date, protist diversity in caves has been scantly investigated, and its knowledge is based on scattered and fragmented published information. However, there have been some reports on the protist diversity from Mexican caves located in the states of San Luis Potosí (Cueva de Los Riscos cave) and Guerrero cave (Osorio-Tafall 1943; Hoffmann et al. 1986; Sigala-Regalado et al. 2011), from Slovenian karst caves (Walochnik and Mulec 2009), and from caves in North America (Holsinger 1966; Barr 1968; Hill et al. 1986; Small et al. 1986). More recently, Baković et al. (2022) investigated the protists of three habitats within the Veternica Cave (Croatia) and reported 47 protist taxa, including 16 ciliates. Regarding Italian caves, Copellotti and Guidolin (1999, 2003) recorded over 100 ciliates from karst caves in Northeast Italy, most of which were new to the Italian checklist (http://www.faunaitalia.it/checklist/). In addition to the classical taxonomic approach, more recently, cave protist diversity has started to be explored by using environmental DNA metabarcoding (eDNA) approaches. A few reports from Appalachian karst caves (USA) and Movile Cave (Romania) were recently published by Reboul et al. (2019) and Cahoon and VanGundy (2022), respectively. Overall, ciliated protists still represent one of the most undersampled and investigated taxa in caves.

The present study shows the presence of a diverse ciliate community in the Frasassi cave system, with 33 ciliate species, (Tables 3, 4, Figs 4–6). The highly diversified site

of PDC, which includes 7 microhabitats, was the most species-rich of the four main sampling sites studied (i.e., PDC, GSO, LVE and RSO), with a total of 32, 11, 5 and 2 ciliate species, respectively (Tables 3, 4, Figs 1–6). The PDC site is composed of a network of different microhabitats, which are connected between themselves exclusively during the wet (rainy) season and mostly separated during the dry season (Fig. 1). Within these microhabitats, the highest species richness recorded at site PCP (17 species) is in contrast with the lower number of ciliate species recorded at site HSS (8 species), which is an extension of site PCP but has a higher sulfur content. It is likely that PCP has a relatively lower sulfur content due to the stream flow, and thus, this progressive dilution of the sulfur concentration along the stream resulted in a richer although less sulfur-tolerant ciliate community. Site SDT, located at the entry point of the cave, had stagnant water with sediments and a bacterial biofilm, providing ideal conditions for the growth of most ciliate species, such as a lack of disturbances related to water flow and an abundant bacterial population as food supply, thus favouring a good richness of ciliate species (n=16). The LDS and LGA sites reached the third and fourth highest numbers of ciliate species (n=13, 11); both sites are characterized by shallow, muddy and stagnant water. The HSS and LDB sites also had low numbers of ciliate species during samplings in October 2009 and September 2011. The HSS is actually a sulfidic water spring with bacterial biofilms, thus allowing the survival of mainly those ciliate species, such as *Cyrtolophosis mucicola*, *Euplotes* sp., *Frontonia leucas*, *Lacrymaria* sp., *Litonotus lamella*, and *Oxytricha* sp., that can possibly tolerate higher sulfur levels (ranging from 322 μM to 542 μM; Table 1) and can graze actively on the bacteria and other microorganisms, including ciliates, associated with the biofilm. LDB is a deep pond with a stagnant yet stratified water column (i.e., carbonate vadose water at the surface and sulfidic water at the bottom), which was expected to have a high species number. However, this was not the case, probably due to the presence of *Coleps hirtus*, a highly active nonselective heterotrophic omnivore, with a high degree of tolerance to stress conditions, such as scarcity of food or extreme pH and temperature variation. Additionally, *C. hirtus* is equipped with extrusomes, which are known to hinder the survival of many other ciliates once cytotoxic substances are released (Buonanno et al. 2014).

The species composition from the ciliate community in PDC was mostly different in October 2009 and September 2011, with a low species similarity between both samplings and only a few species recorded on both occasions ("resident species"). However, the comparison of taxonomic (at the order level) and trophic groups present in PDC on both dates suggests that the individual species could play similar roles within each taxonomic or trophic group, keeping the ciliate community stable over time.

From the other three main sampling sites, i.e., LVE, RSO and GSO, the lowest number of species was recorded at RSO, with only two species present, *Paramecium caudatum* and *Oxytricha* sp. When compared to the other sites, RSO is the most oligotrophic habitat, characterized by having clear water, sulfur content ranging from 109 μM to 240 μM (Table 1), and absence of biofilms. It is likely that the presence of bacterial, flagellate and/or algal populations adapted to these environments could justify the presence of bacteri-

vores or phytobacterivore ciliates such as *Paramecium* and *Oxytricha*. Similar conditions were reported by Foissner (1997) in a study of the ciliates in four clear water rivers in Germany, where the low abundance of ciliates was attributed to the shortage of nutrients. The samples collected in LVE, a deep stratified lake, presented five ciliate species: *Coleps hirtus*, *Oxytricha* sp., *Vorticellides aquadulcis*, *Cyrtolophosis mucicola* and *Urocentrum turbo*. Eleven species were present in GSO samples, namely, *Litonotus lamella*, *Cyrtolophosis mucicola*, *Euplotes aediculatus*, *Brachonella* sp., *Urocentrum turbo*, *Chilodonella uncinata*, *Caenomorpha* sp., *Coleps hirtus*, *Oxytricha* sp., *Vorticellides aquadulcis* and *Aspidisca turrita* (Table 3, Figs 3–6). The GSO site is represented by a small water pool characterized by a sulfur content ranging from 118 μM to 415 μM (Table 1).

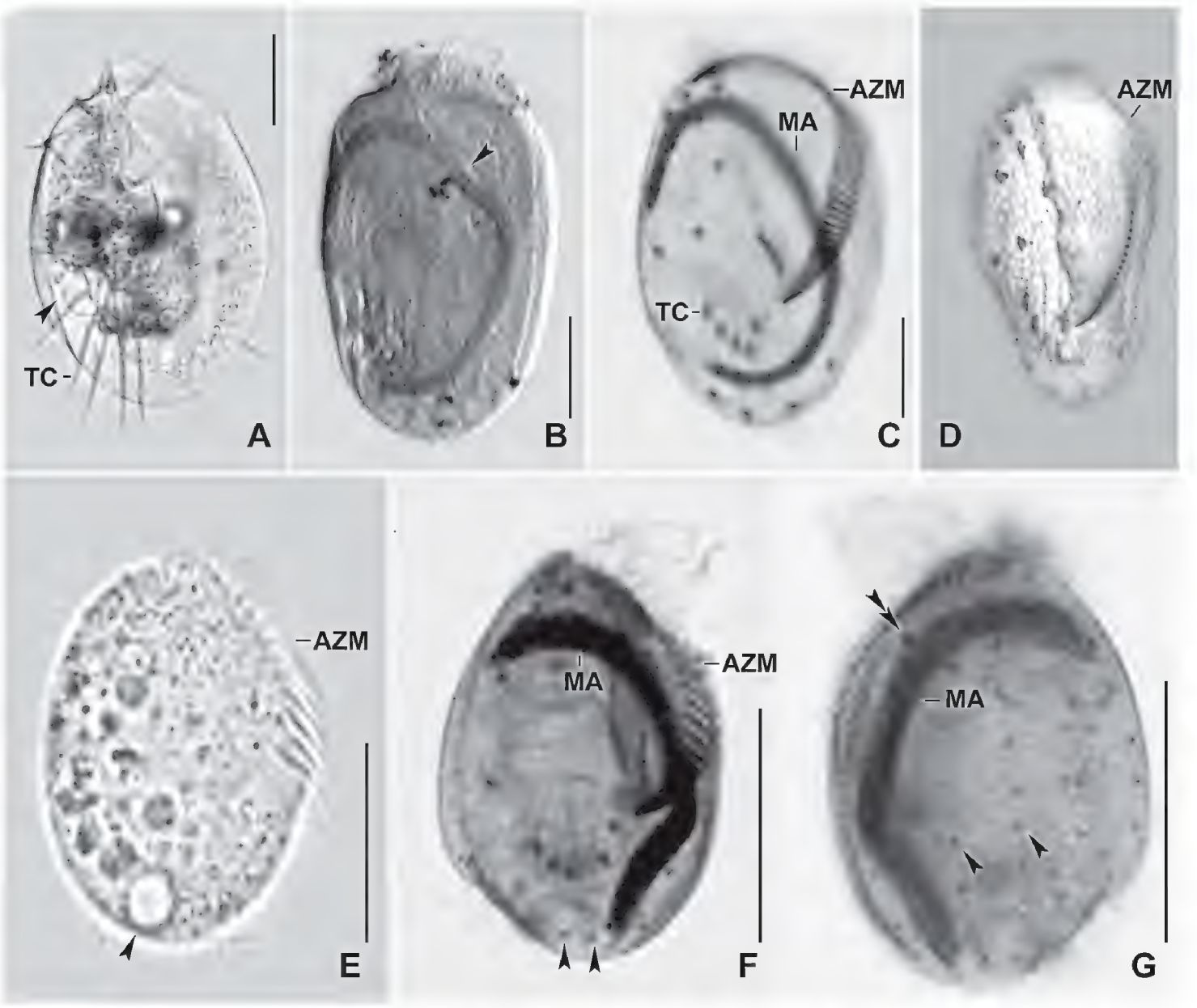

Figure 6. Photomicrographs of live **A, E** silver impregnated cells **B–D, F, G** from GSO **A–D** and PDC **E–G**. **A–D** representative specimens of *Euplotes aediculatus*. Note the contractile vacuole in a live specimen (arrowhead in **A**), the horse shoe shaped macronucleus (arrowhead) **B** and the infraciliature and silverline pattern **C, D**. **E–G** *Euplotes* sp. live **E** and ventral and dorsal views of protargol impregnated specimens **F, G**. Arrowhead in **E** points to the contractile vacuole. Arrowheads in **F** denote the caudal cirri. Double arrowhead in **G** points to the micronuclei and arrowheads to the dorsal kinety rows. **AZM** = adoral zone of membranelles; **MA** = macronuclear nodule; **TC** = transverse cirri. Scale bars: 40μm (**A–C**), 20μm (**E–G**).

It was observed that some species were "permanent resident species", i.e., they were recovered from the same site on different sampling campaigns, including *Brachonella* sp., *Chilodonella uncinata*, *Coleps hirtus*, *Cyrtolophosis mucicola*, *Oxytricha setigera*, *Oxytricha* sp., *Paruroleptus* sp., and *Vorticellides aquadulcis*. This indicates that these species might be adapted to that particular habitat with regard to maintaining their population under fluctuating environmental conditions. Furthermore, a curious observation was that the high density of individuals of *Oxytricha* sp. found in PDC covered the whole gastropod shell of *Islamia sulfurea* (Bodon and Cianfanelli 2012), probably feeding on the bacteria involved in their decomposition and depositing resting cysts, which is a strategy to protect themselves from predators as well as excysts when conditions are favourable in a more protected environment. In this regard, a ciliate cyst represents a key food resource for protists as well as invertebrates (Bharti et al. 2020).

Flagellates are more abundant than ciliates and are often mixotrophic, occupying both planktonic and benthic levels (Corliss 2002). The low diversity of flagellates, amoebas, and heliozoans recorded in the present study is because the samples were processed with the main focus on detecting ciliate species. Flagellates and heliozoans found in the water/sediment samples from the Frasassi caves were benthic, and as soon as they appeared in the culture, they grew rapidly, overcoming other protist species.

Furthermore, this study led to the discovery of 14 species that are new to Italian ciliate records, i.e., nearly 40% of the total species identified (Table 3). This indicates that a large gap still exists in published Italian ciliate diversity studies. It is believed that efforts based on an integrative approach (i.e., by molecular, morphological, and behavioural approaches) to study actual ciliate diversity will enrich the Italian list as well as the list of known ciliates worldwide (Bharti et al. 2018; Serra et al. 2020). This is also evident from recent studies where several new species have been documented from Italian soils (Bharti et al. 2014, 2015, 2016, 2017; Kumar et al. 2014).

Cryptic species

Cryptic ciliate species were recovered by enriching samples to encourage the growth of dormant ciliates. The number of ciliate species increased in the enrichment cultures. Previous studies have shown the potential significance of the local 'seedbank' of ciliates. For example, dilution of water from a hypersaline lagoon (Esteban and Finlay 2003) yielded typical freshwater ciliate species, while in a study of a freshwater pond (Fenchel et al. 1997), the number of observed ciliate species increased from 20 to 135 following the use of enrichment cultures. Similarly, the observation of freshly collected cave samples showed few active ciliate species (Tables 5, 6), whereas following enrichment, several other species appeared, mainly those that possess the ability to form resting cysts, such as hypotrich ciliates, while the others were possibly present in one or two specimens that increased their abundance post enrichment. The ability of a

given ciliate species to thrive out of its preferred ecological conditions indicates that if it is dispersed to a different location to which it is not apparently suited, it may adapt and succeed, explaining the global distribution of that species (Finlay 2002). Sometimes, such species fail to produce substantial populations in the new habitat or cannot withstand the environmental or ecological conditions of the new habitat, entering a dormant stage, which results in the so-called 'seedbank' of cryptic species within that habitat (Esteban and Finlay 2010).

Table 5. Distribution of ciliate species found in PDC in October 2009 (upper line) and September 2011 (lower line). + indicates presence of the species.

S. No.	Species	PDC						
		SDT	LDS	HSS	PCS	PCP	LGA	LDB
1.	*Aspidisca turrita*						+	
						+		
2.	*Brachonella* sp.		+			+		
			+			+		
3.	*Chilodonella uncinata*				+			
					+	+		
4.	*Coleps hirtus*						+	+
					+	+		+
5.	*Colpoda inflata*						+	+
6.	*Cyrtolophosis mucicola*		+	+	+		+	+
					+	+		+
7.	*Dileptus* sp.						+	
					+	+		
8.	*Euplotes* sp.						+	
9.	*Litonotus lamella*						+	
			+	+				
10.	*Oxytricha setigera*					+		
				+		+		
11.	*Oxytricha* sp.		+					
			+	+				+
12.	*Paracolpoda steinii*						+	
13.	*Paruroleptus* sp.					+		
						+		
14.	*Pelagothrix* sp.						+	
15.	*Spathidium* sp.						+	
						+		
16.	*Tachysoma pellionellum*							+
17.	*Tetrahymena pyriformis*							
						+		+
18.	*Vorticellides aquadulcis*				+		+	
			+		+	+		
	Total number of species	–	**5**	**4**	**5**	**11**	**11**	**6**

Table 6. Occurrence of ciliates species after enrichment of the samples collected in September 2011 in PDC with autoclaved rice and the green algae *Chlorogonium elongatum*. + indicates presence of the species.

Species	LDS				HSS				PCS				PCP				LDB			
Days 1–7	1	3	5	7	1	3	5	7	1	3	5	7	1	3	5	7	1	3	5	7
Aspidisca turrita														+						
Chilodonella uncinata									+		+	+		+	+	+				
Coleps hirtus											+		+	+			+	+	+	+
Cyrtolophosis mucicola		+	+	+	+					+	+	+	+	+		+			+	+
Dileptus sp.												+	+							
Litonotus lamella				+				+												
Oxytricha setigera							+									+				
Oxytricha sp.				+				+										+		+
Paruroleptus sp.																+				
Spathidium sp.														+						
Tachysoma pellionellum																			+	+
Tetrahymena pyriformis														+	+	+		+	+	+
Vorticellides aquadulcis				+							+	+			+					

Adaptations of ciliates species to cave habitats

This study was primarily conducted to obtain an overview of the protist ciliate diversity in the different investigated sites of the Frasassi cave, including possible differences at the behavioural and morphological levels between the cave-dwelling ciliate species and their noncave-dwelling conspecifics. It has been observed that organisms living under sulfidic conditions manifest different morphological, behavioural and physiological adaptations compared to nonsulfidic subsurface animals as a result of different environmental stresses (Engel 2007). In the present study, some of the species isolated from the cave could not be cultured in the laboratory. For example, a cave strain of *Urocentrum turbo* was found to be extremely photosensitive, and the cells began to burst when they were exposed to light; such an answer likely has an adaptive value as the result of microevolution in perpetually dark habitats (Fig. 7). To date, similar behaviour, although not with such a dramatic response, has been described in a few other ciliated protists, such as *Stentor coeruleus*, which escapes from areas of high light intensity as an antipredation strategy (Cadotte et al. 2007), and the psychrophilic ciliate *Euplotes focardii*, which inhabits the shallow marine coastal sediments of Antarctica (La Terza et al. 2001). In this extremophile ciliate, which shares with *U. turbo* a perpetually dark habitat, exposure to visible light is able to induce the expression of its heat shock protein (hsp) 70 genes as a defensive response (La Terza et al. 2004, 2007; Fulgentini et al. 2015). However, no photosensitivity was recorded in noncave-dwelling populations studied from Italy and India, although the Italian noncave-dwelling population showed an absence of trichocysts. Similar observations were reported by Stoeck et al. (2007) in *Urocentrum turbo* isolated from a meromictic anoxic alpine lake (Alatsee, Germany), showing high similarity in morphology and gene sequences with described populations but lacking trichocysts, a conspicuous and characteristic feature of this species. The presence of trichocysts in the Frasassi cave ciliate population might be explained considering the extremophile environment and the need to protect themselves against predation, e.g., by other ciliate taxa such as haptorid species

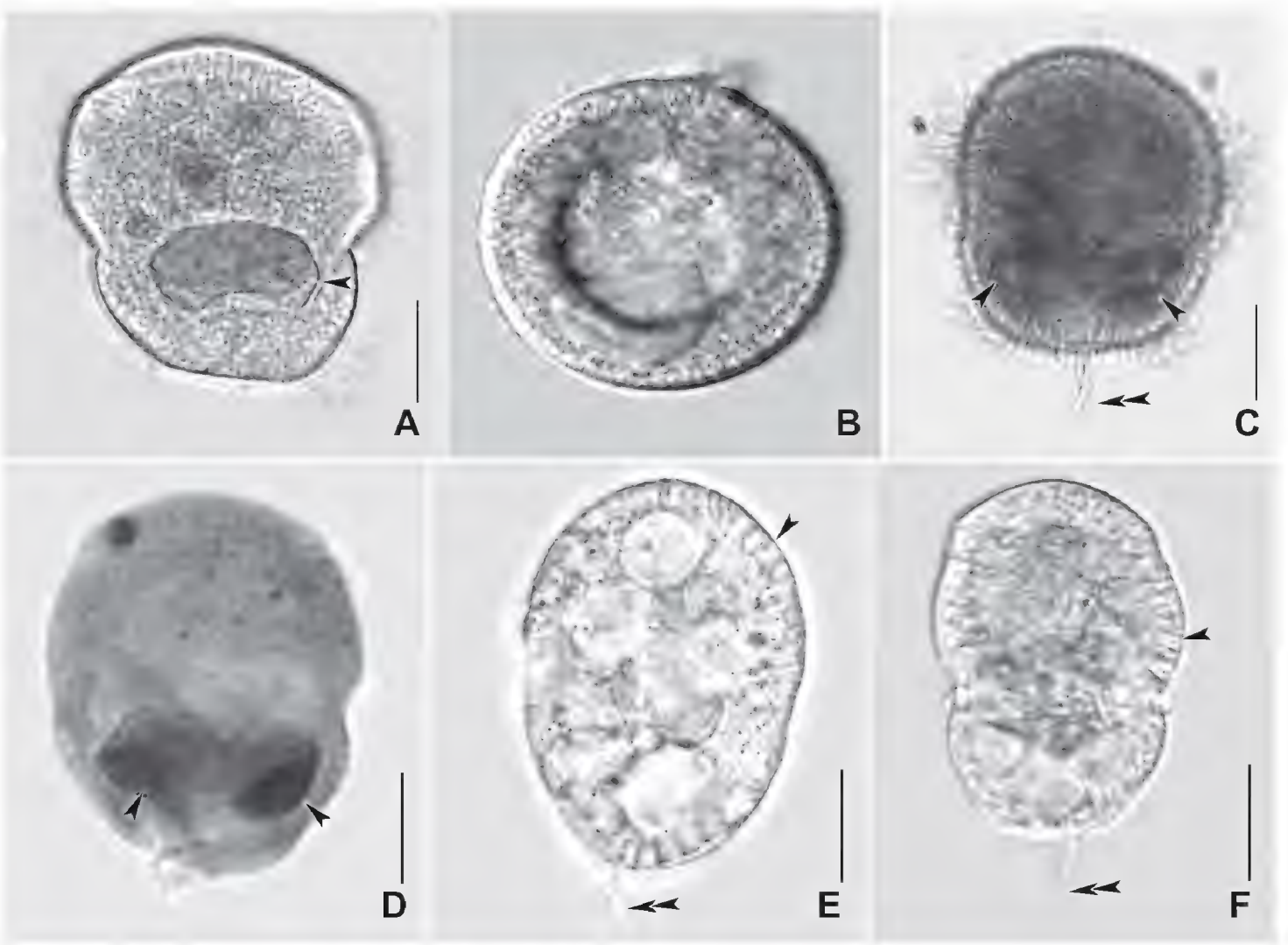

Figure 7. Photomicrographs of live **A, B, E, F** bouin's fixed **C** and protargol-impregnated **D** *Urocentrum turbo* from PDC **A–C** and Sentino River population **D** and Indian populations **E, F**. **A** a live specimens of *Urocentrum turbo* **B** specimen about to burst after forming atypical structure as consequence of light exposure **C** specimen fixed with bouin's fixative to avoid bursting (arrowheads mark the macronucleus) and record details **D** a non-cave dwelling population collected outside the cave (Sentino River) of the Frasassi cave, arrowheads marks the macronucleus. Note that the Sentino River specimens were found to be resistant to light exposure though deprived of trichocysts. **E, F** the Indian populations were found to be resistant to light exposure and possessed the trichocysts (arrows), double arrowheads mark the caudal cirri. Scale bars: 25 μm.

from the genera *Dileptus*, *Lacrymaria* and *Spathidium* or the prostomatid *Coleps hirtus*, a nonselective heterotrophic omnivore. In this regard, this is a winning strategy because *U. turbo* is an almost ubiquitous species that has been isolated from all the investigated sites with the only exception of RSO.

It has been demonstrated that some morphological features, such as the dorsal thorn in *Aspidisca turrita* and the dorsal ridges in *A. costata*, are inducible defences formed when the organism is exposed to chemical cues produced by certain predators but are lost or significantly reduced in the absence of these cues (Wicklow 1997). The Frasassi cave strain of *Aspidisca turrita* was found to possess two to three dorsal spines (with respect to its noncave-dwelling conspecific), which could represent a temporary morphological defensive trait induced by the presence of potential predators dwelling inside the cave.

The response of ciliate species to various stress conditions can be extremely variable, i.e., formation of a dormant form (cysts), reduction in size, induction of conjugation, and shedding of the oral apparatus and ciliature, among others. Wan et al. (2019) reported

that the *Stentor coeruleus* shows induction of reorganization of the oral apparatus as well as the ciliature (ventral and dorsal) in response to external stressful stimuli, such as primarily chemical exposures. In this regard, the frequent reorganization events (nearly 20 out 100 specimens analysed) observed in the cave-dwelling population *of Euplotes aediculatus* could be attributed to self-repair mechanisms due to the damage caused by the chemical exposure within the cave (Fig. 8). We suppose that *E. aediculatus* could be sensitive to some of the chemicals (i.e., sulfide) in the cave that are able to cause damage, which in turn triggers the initiation of the formation of a new set of oral apparatus and ciliature. In-depth studies are required to understand the timing and mechanistic aspects of reorganization of ciliature resulting from chemically exposed cells of *E. aediculatus* in the cave.

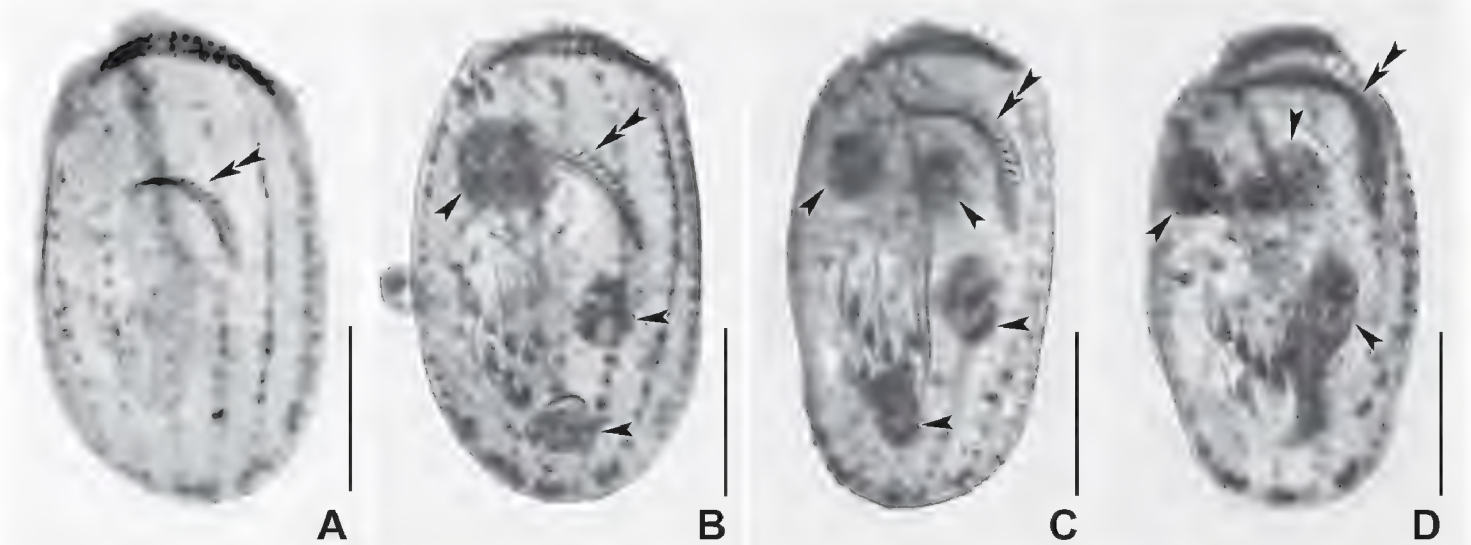

Figure 8. *Euplotes aediculatus* specimens under different stages of reorganization. Double arrowheads point to the newly formed adoral membranelles and arrows to the fragmented macronucleus. Scale bars: 40 μm.

Furthermore, it was observed that in the raw cultures, *C. hirtus* increased their number to such an extent that it utilized most of the resources available, thereby hindering the growth of many other ciliates in the same culture medium. The toxicity of *Coleps hirtus* extrusomes on several ciliates was described by Buonanno et al. (2014), who reported that *C. hirtus* performs multiple attacks (pack hunting) on its prey, administrating a lethal concentration of toxins that cause its rapid immobilization for easier predation. For example, extrusomes can be used by this ciliate to kill much larger organisms, such as young larvae of zebra fish (Mazanec and Trevarrow 1998). This feeding strategy differs from that of hypotrich ciliates, which capture the food particles by generating a water current using the adoral zone of membranelles, which carries the food through the buccal field until it is engulfed in single or several food vacuoles. *Coleps hirtus* is described as an omnivorous feeder that is attracted by dying or dead organisms, including cells of its own cell type (Falkner 1951; Seravinand Orlovskaja 1977). However, in the present work, we observed a previously unseen cannibalistic behaviour in *C. hirtus*, consisting of the intraspecific aggression of this species, i.e., it actively hunts its own living conspecific (Fig. 9). The significance of this feeding behaviour for the strains of *C. hirtus* found in

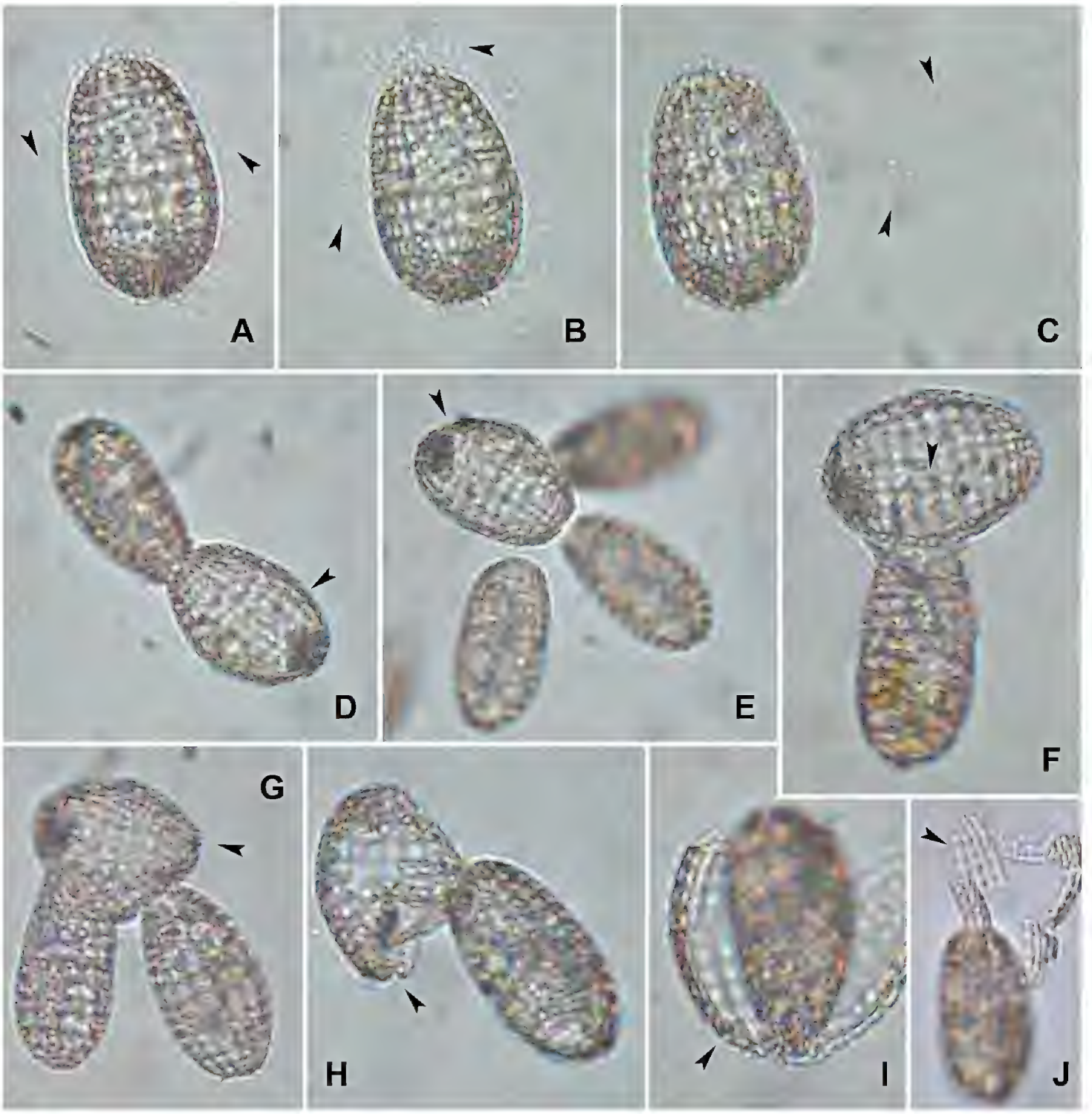

Figure 9. Photomicrographs showing the details of the cannibalistic behaviour of *Coleps hirtus* **A** a static specimen with straight cilia due to multiple attacks by the predatory cells, arrowheads mark the cilia **B, C** same specimens after few seconds with detached cilia (arrowheads) **D, E** the predatory cells tries to feed on the dead cell, arrowheads in **D, E, H–J** points to the dead cell **F** the feeding starts with sucking of the cytoplasm and nuclear apparatus, arrowhead marks the macronuclear nodule **G–J** the predatory cells then feed on the calcified armours plates by breaking them into small pieces. The average length of live specimens of *Coleps hirtus* Frasassi cave population is 50 μm.

the Frasassi caves could be a result of adaptation to these harsh environments, as it provides them with an opportunity to maintain their populations for longer periods, particularly in oligotrophic environments. Although this behaviour has been least studied in microbes, especially single-celled protist ciliates, it is well known in arachnids (spiders), crustaceans (hermit crabs), amphibians (toads and salamanders) and reptiles (lizards), among others, as it has a rather deep impact on populations by affecting their fitness and dynamics (Arnott and Elwood 2008; Cooper et al. 2015; Melotto et al. 2019).

Coleps hirtus has been widely used as a model organism by ciliate ecologists due to its wide distribution; high range of tolerance to temperature, starvation, and low oxygen concentration; broad food spectrum; etc. (Foissner et al. 1999; Pfister et al. 2002). Our observations are based on several populations of *C. hirtus* isolated from different locations in the Frasassi cave complexes in Italy as well as in small ponds near the bank of the Ganges River (Hooghly), Kolkata, West Bengal, India.

Molecular sequence comparison based on the 18S rRNA gene between cave-dwelling ciliate species and their noncave-dwelling conspecifics showed no appreciable differences, since the similarity values were, in most cases, equal to 99% with the exception of C*oleps hirtus*, which shared a similarity value of 98% with both *C. viridis* and *C. hirtus* (Table 7).

Table 7. Molecular (18S rRNA gene) characterizations of selected cave-dwelling species with non-cave-dwelling counterpart.

Species/NCBI Accession number	Sampling site	18S rDNA sequence length (bp)	Highest identity*	Similarity value (%)
Coleps hirtus hirtus (KF177278)	LVE, PDC (PCS, LGA, LGB)	1775	*Coleps viridis* (MT253681) / *Coleps hirtus* (MT253687)	98
Climacostomum virens (ON678183)	PDC (SDT)	1711	*Climacostomum virens* (X65152)	99
Euplotes aediculatus (ON678547)	LVE, PDC (LDS)	1874	*Euplotes aediculatus* (EU103618)	99
Euplotes sp. (ON678276)	LVE, PDC (SDT, LDS, HSS, LGA)	1600	*Euplotes elegans* (DQ309868)	99
Vorticella picta (ON678258)	PDC (SDT)	1712	*Vorticella fusca* (DQ190468)	98
Urocentrum turbo (ON678277)	GSO	1660	*Urocentrum turbo* (AF255357)	99

* Non-cave-dwelling conspecific.

Regarding the latter point, it is worth mentioning that *Coleps hirtus* has been studied thoroughly recently by Pröschold et al. (2021), which shows that this species possesses high phenotypic plasticity and low genetic variability, thereby suggesting a future revision of the species concept for the genus *Coleps*. *Coleps hirtus* and *Colep viridis* are mainly separated by the presence of endosymbiont algae in the latter. Pröschold et al. (2021) reported that the presence or absence of endosymbiont algae could possibly be a weak species identification characteristic, at least in the genus *Coleps*, and that the mutual relationship between *Coleps* and algae is facultative. In this regard, the cave population of *Coleps hirtus* did not show the presence of algae, and it could be assumed that this may be due to the absence of light resulting in the absence of symbiosis. Furthermore, the sequence similarity is 98% between the cave and German populations of *Coleps hirtus* described by Pröschold et al. (2021). In this regard, we believe that the genus *Coleps* needs further detailed investigations, based on several populations worldwide, for observations regarding phenotypic plasticity. Thus, we still proceed with the old concept and consider *Coleps hirtus* and *C. viridis* as separate species until further investigation.

Conclusion

Overall, the present study provides a baseline survey of the diversity of cave-dwelling ciliated protists from the different microhabitats of the Frasassi cave ecosystem and describes some peculiar morphological and behavioural differences with their noncave-dwelling conspecifics that have not been substantiated at the molecular level. Thus, these results open the way for further investigations to be conducted *via* integrative taxonomic approaches (i.e., morphology, ontogeny, ecology, behaviour, eDNA, etc.) to better decipher the cryptic diversity of these almost neglected and undersampled taxa of eukaryotic microorganisms and their functional roles within the chemoautotrophic cave ecosystem of Frasassi.

Acknowledgements

This research was supported by the Research fund from University of Camerino (FAR) to ALT. SK and DB were financially supported by a "Young Indian Research Fellowship" through the Italian Minister of University and Research (MUR) to ALT. Financial support by the Department of Science and Technology (DST Project no CRG/2019/004203 to SK), India and internal program of Zoological Survey of India, Kolkata, India, and the non-profit Association "Le Montagne di San Francesco" in Coldigioco, is greatly acknowledged. We would like to thank Sandro Mariani (Gruppo Speleologico Cai di Fabriano); Simone Cerioni Gruppo Speleologico di Genga), Jenn Macalady (Pennsylvania State University), and Sharmishtha Dattagupta (Georg-August-Universität, Göttingen), for collecting samples from RSO and GSO. The authors greatly thank the two reviewers Prof. R. Mayén-Estrada and Prof. I. Sigala-Regalado for providing us constructive and helpful comments that improved the quality of the manuscript.

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Supplementary material 1

Cannibalistic behaviour of *Coleps hirtus* on other conspecific living individuals (Video 1)

Authors: Daizy Bharti, Santosh Kumar, Federico Buonanno, Claudio Ortenzi, Alessandro Montanari, Pablo Quintela-Alonso, Antonietta La Terza

Data type: Multimedia

Explanation note: *Coleps hirtus* performing multiple attacks (pack hunting) on its prey, administrating a lethal concentration of toxins that cause its rapid immobilization for easier predation.

Link: https://doi.org/10.3897/subtbiol.44.96545.suppl1

Supplementary material 2

Cannibalistic behaviour of *Coleps hirtus* on other conspecific living individuals (Video 2)

Authors: Daizy Bharti, Santosh Kumar, Federico Buonanno, Claudio Ortenzi, Alessandro Montanari, Pablo Quintela-Alonso, Antonietta La Terza

Data type: Multimedia

Explanation note: *Coleps hirtus* feeding on immobilization individual.

Link: https://doi.org/10.3897/subtbiol.44.96545.suppl2

Supplementary material 3

Cannibalistic behaviour of *Coleps hirtus* on other conspecific living individuals (Video 3)

Authors: Daizy Bharti, Santosh Kumar, Federico Buonanno, Claudio Ortenzi, Alessandro Montanari, Pablo Quintela-Alonso, Antonietta La Terza

Data type: Multimedia

Explanation note: *Coleps hirtus* feeding on immobilization individual.

Link: https://doi.org/10.3897/subtbiol.44.96545.suppl3

Supplementary material 4

Cannibalistic behaviour of *Coleps hirtus* on other conspecific living individuals (Video 4)

Authors: Daizy Bharti, Santosh Kumar, Federico Buonanno, Claudio Ortenzi, Alessandro Montanari, Pablo Quintela-Alonso, Antonietta La Terza

Data type: Multimedia

Explanation note: *Coleps hirtus* feeding on immobilization individual. The cytoplasm has already been fed up and the feeding on armour plates is in process.

Link: https://doi.org/10.3897/subtbiol.44.96545.suppl4